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Journal of Ethnopharmacology 48 (1995) 85–88

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Antigonorrhoeal activity of plants used in Guatemala for the treatment of sexually transmitted diseases[1]

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Abstract

Plants popularly used in Guatemala for the treatment of gonorrhoea were macerated in 50% alcohol and the tincture tested for *in vitro* activity against *Neisseria gonorrhoeae* using strains isolated from symptomatic patients and confirmed by standard bacteriological procedures. From 46 plants investigated, 13 (28.3%) showed evident inhibition zones (>9 mm), seven (15.2%) showed small activity (6.1–8.9 mm) and 26 (56.5%) showed no activity; nine of these plants inhibited five strains of *N. gonorrhoea* freshly isolated. The most active plants of American origin were: bark of *Bixa orellana* fruits of *Parmentiera edulis*, leaf of *Diphysa robinioides*, *Eupatorium odoratum*, *Gliricidia sepium*, *Physalis angulata*, *Piper aduncum* and *Prosopis juliflora*, root of *Casimiroa edulis*, and whole *Clematis dioica*.

Keywords: Antigonorrhoeal activity; Guatemala; *Bixa orellana*; *Clematis dioica*; *Eupatorium odoratum*; *Parmentiera edulis*

1. Introduction

Gonorrhoea is a sexually transmitted disease with a growing frequency in developed and developing countries. Treatment is done with antibiotics, although the appearance of resistant strains is becoming more frequent (Schoolnick et al., 1985). Mesoamerica is well known for its biodiversity but also as in Guatemala, the traditional utilization of medicinal plants for healing is frequent (Orellana, 1987), but studies to validate the properties claimed are still limited. The need to validate the traditional use of plants in Mesoamerica and the Caribbean (Robineau, 1991) and the search for antimicrobial substances with less toxicity and more available by the population, motivated several screening studies of the antimicrobial activity of plants popularly used against pathogenic bacteria (Cáceres et al., 1987; 1990;

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1991a), yeast (Girón et al., 1988; Cáceres et al., 1991b) and dermatophytes (Cáceres et al., 1991c; 1991d).

Traditional use of herbal preparation is frequent for treating sexually transmitted diseases; in Central America and the Caribbean 101 plants are claimed to be useful in traditional medicine for the treatment of gonorrhea (Morton, 1981). In this study, 46 tinctures from 44 plants used for the treatment of sexually transmitted diseases are screened for activity against *Neisseria gonorrhoeae*.

2. Materials and methods

2.1. Plants and extracts

All plants were collected from Highland and Western regions of the country according to endemicity, references from the literature and previous contacts by our personnel. Specimens were identified at the Herbarium of the Faculty of Agronomy, and the voucher specimens of the plants were deposited in CEMAT-FARMAYA Ethnobotanical Herbarium, Guatemala. Two samples were provided by Enda-Caribe and their voucher samples are deposited in the Botanical Garden of Santo Domingo, Dominican Republic.

The plant material was air dried in shade and powdered. Tinctures were prepared by weighing 10 g of plant, macerated with 90 ml of 50% ethanol for 5 days with daily agitation and filtration with Whatman paper No. 2. Absorbent paper disks (6 mm diameter, 0.6 mm thick) were soaked with 50 μl of the tincture, equivalent to 50 mg of starting dry vegetal material and dried in a laminar flow hood.

2.2. Antigonorrheal testing

A penicillin-resistant strain of *N. gonorrhoeae* was isolated in Thayer-Martin Agar from a symptomatic patient, characterized in the bacteriology laboratory and an innoculum standardized to MacFarland nephelometer tube No. 0.5. This suspension was innoculated over plates containing Mueller-Hinton chocolate agar with 5% sheep red blood cells and IsoVitalex, quintuplicate of the disks were placed randomly and the plates incubated for 24 h at 35°C in a candle jar (10% CO_2). Inhibition zones were measured in millimeters with a transparent ruler and compared against the solvent, the test was validated previously with penicillin impregnated disks and activity established according to previous work (Cáceres et al., 1990). Out of 16 plants with positive, intermediate and negative results, the spectrum of inhibition was determined by challenging disks with 50 μl of the tincture against five strains of *N. gonorrhoeae* freshly isolated from clinical patients, characterized in the same way and showing different patterns of antibiotic resistance.

3. Results and discussion

In 46 plant extracts, 13 (28.3%) showed evident inhibition zones (>9 mm), 7 (15.2%) intermediate activity (6–9 mm) and 26 (56.5%) showed no activity (<6 mm), in *Bixa orellana* and *Spondias mombin* two organs of the plant were studied (Table 1). The plants of American origin which showed better activity against *N. gonorrhoeae* were: *Bixa orellana*, *Casimiroa edulis*, *Clematis dioica*, *Diphysa robinioides*, *Eupatorium odoratum*, *Gliricidia sepium*, *Parmentiera edulis*, *Physalis angulata*, *Piper aduncum* and *Prosopis juliflora*.

From the plant tinctures screened, a group of 16 were selected for spectrum inhibition studies (12 positive, one intermediate and three negative) with five strains of *N. gonorrhoeae*. Nine of twelve positive extracts inhibited the five strains (100%), three positive extracts and one intermediate inhibited four (80%) strains. The three negative extracts showed no activity against the five strains.

Information on medicinal plants used for the treatment of gonorrhea and reports on antigonorrhoeal activity of plants is very scarce. The regional Atlas of medicinal plants by Morton (1981) includes 101 plants used for the treatment of gonorrhoea, 25 were screened in this study, six (24%) showed antigonorrhoeal activity. An ethnobotanical survey in Rwanda showed 100 plants used for the treatment of infection, 23 are used for the treatment of gonorrhoea (Boily and van Puyvelde, 1986); from 25 plant extracts used in this preparations, 16 (65.6%) showed some activity against *N. gonorrhoeae*; from 23 indigenous drugs, 17 (74%) contain one or more plants with activity

Table 1
Antigonorrheal activity of tinctures of Guatemalan plants

Family	Scientific name	Voucher number[a]	Part used	Inhibition (mm)	Spectrum (%)
Acanthaceae	*Justicia spicigera* Schlecht.	CF-327	Leaf	8.0 ± 0.1	N.D.
Amarillidaceae	*Agave americana* L.	CF-378	Root	6.0 ± 0.1	N.D.
Anacardiaceae	*Spondias mombin* L.	CF-325	Bark	7.6 ± 0.5	N.D.
	Spondias mombin L.	CF-325	Leaf	6.0 ± 0.1	N.D.
Asclepiadaceae	*Asclepias curassavica* L.	CF-149	Leaf	6.0 ± 0.1	N.D.
Bignoniaceae	*Parmentiera edulis* DC	CF-330	Fruit	13.9 ± 1.3	100
Bixaceae	*Bixa orellana* L.	CF-319	Root	6.0 ± 0.1	0
	Bixa orellana L.	CF-319	Leaf	17.4 ± 0.5	100
Burseaceae	*Bursera simaruba* Sarg.	CF-225	Leaf	6.0 ± 0.1	N.D.
Cactaceae	*Hylocereus undatus* Britt. et Rose	CF-221	Leaf	6.0 ± 0.1	N.D.
Caricaceae	*Carica papaya* L.	CF-227	Root	12.5 ± 0.6	100
Commelinaceae	*Zebrina pendula* Schnizl.	CF-178	Whole	6.0 ± 0.1	N.D.
Compositae	*Acanthospermum hispidum* DC	CF-331	Leaf	11.3 ± 0.5	100
	Ageratum conyzoides L.	CF-328	Leaf	8.0 ± 0.1	N.D.
	Eupatorium odoratum L.	CF-321	Leaf	11.0 ± 0.1	100
	Tagetes lucida Cav.	CF-164	Leaf/flower	8.4 ± 0.4	N.D.
Euphorbiaceae	*Pedilanthus tithymaloides* Poit.	CF-176	Leaf	6.0 ± 0.1	N.D.
Graminea	*Saccharum officinarum* L.	CF-464	Stalk	6.0 ± 0.1	0
	Zea mays L.	CF-240	Stigma	6.0 ± 0.1	N.D.
Labiatae	*Satureja browneii* Briq.	CF-192	Whole	6.0 ± 0.1	N.D.
Leguminosae	*Acacia farnesiana* Willd.	CF-103	Leaf	6.0 ± 0.1	N.D.
	Caesalpinia pulcherrima Swartz	CF-246	Root	6.0 ± 0.1	N.D.
	Cajanus cajan Millsp.	CF-290	Leaf	8.0 ± 0.1	N.D.
	Cassia occidentalis L.	CF-249	Leaf	6.0 ± 0.1	N.D.
	Diphysa robinioides Benth.	CF-209	Leaf	9.6 ± 0.2	100
	Gliricidia sepium Steud.	CF-233	Leaf	10.2 ± 0.6	80
	Mucuna urens DC	CF-332	Seed	6.0 ± 0.1	N.D.
	Prosopis juliflora DC	CF-334	Leaf	9.6 ± 0.2	100
Malvaceae	*Abelmoschus esculentus* Moench.	CF-336	Fruit	10.0 ± 0.1	80
	Sida rhombifolia Swartz	CF-259	Leaf	6.0 ± 0.1	N.D.
Moraceae	*Cecropia peltata* L.	CF-333	Leaf	6.0 ± 0.1	N.D.
Myrtaceae	*Pimenta racemosa* var. *ozua* Landum	FJ 60/90	Leaf	6.0 ± 0.1	N.D.
	Psidium guajava L.	CF-105	Leaf	6.0 ± 0.1	N.D.
Nyctaginaceae	*Mirabilis jalapa* L.	CF-212	Leaf	6.0 ± 0.1	N.D.
Palmaceae	*Cocos nucifera* L.	CF-270	Root	6.0 ± 0.1	0
Piperaceae	*Piper aduncum* L.	CF-234	Leaf	10.4 ± 0.8	80
	Piper auritum HBK.	CF-273	Leaf	6.0 ± 0.1	N.D.
Portulacaceae	*Portulacca oleracea* L.	CF-324	Leaf	6.0 ± 0.1	N.D.
Ranunculaceae	*Clematis dioica* L.	CF-201	Whole	13.2 ± 0.9	100
Rhizophoraceae	*Rhizophora mangle* L.	CF-323	Bark	7.7 ± 0.3	N.D.
Rubiaceae	*Chiococa alba* Hitchc.	FJ 79/90	Root	6.0 ± 0.1	N.D.
Rutaceae	*Casimiroa edulis* Llave et Lex	CF-316	Root	12.3 ± 1.7	100
Sapotaceae	*Chrysophyllum cainito* L.	CF-320	Bark	6.0 ± 0.1	N.D.
	Manilkara achras Royen	CF-329	Bark	8.5 ± 0.1	80
Smilacaceae	*Smilax lundellii* Killip et Morton	CF-208	Rhizome	6.0 ± 0.1	N.D.
Solanaceae	*Physalis angulata* L.	CF-335	Leaf	9.2 ± 0.3	N.D.
Sterculiaceae	*Guazuma ulmifolia* Lam.	CF-332	Bark	6.0 ± 0.1	N.D.
Urticaceae	*Urera baccifera* Gaud.	CF-326	Root	6.0 ± 0.1	N.D.

Inhibition: mean ± S.D.(in mm). Spectrum: % of inhibited strains; N.D., not done.
[a]Herbarium: CF, CEMAT-FARMAYA; FJ, Francis Jiménez, Botanical Garden of Santo Domingo.

against gonorrhoea (van Puyvelde et al., 1983). In a study of 31 Tanzanian plants, 13 (42%) extracts showed activity against *N. gonorrhoeae* (Chhabra and Uiso, 1991). In a study from Zimbabwe, the chlorofom extract of *Plumbago zeylanica* showed activity against penicillin and non-penicillin resistant strains of *N. gonorrhoeae* (Gundidza and Manwa, 1990). Only one plant (*Cajanus cajan*) is common to two of these studies and ours, good activity was demonstrated in the Rwandese study, but moderate activity in our study.

Our results indicate that the leaves of *B. orellana* and the bark of *S. mombin* showed some activity, but the roots of *B. orellana* and the leaves of *S. mombin* showed no activity, although ethnobotanical information indicated the use of these parts for the treatment of gonorrhoea (Morton, 1981). Literature review indicated that this activity has not been previously reported for these plants.

Evidence is presented that several plants popularly used in Guatemala for the treatment of sexually transmitted diseases have *in vitro* activity against *N. gonorrhoeae*. Spectrum inhibition studies showed that most of the positive plants have a relatively broad spectrum, since they are active against five strains of *N. gonorrhoeae*.

Toxicity studies will continue, as well as further studies in their spectrum of activity against other microorganism. These results are encouraging and urged for the need of further research to determine the chemical structures of the active principles responsible of the activity and other pharmacological characteristics that could explain the mode of action and provide the preclinical information for further clinical application.

Acknowledgements

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Antimicrobial Activity of Nine Common Plants in Kerala, India

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Kerala, India 673635

ABSTRACT

Nine common plants, namely, Aegle marmelos *(L.) Corr.,* Leucas indica *(L.) Vatka,* Murraya koenigii *(L.) Spreng.,* Tamarindus indica *(L.)* Pachyptera alliacea *(Lam.) A. Gentry,* Eupatorium odoratum *(L.),* Moringa oleifera *(Lamk.),* Cinnamomum veerum *(Preal) and* Cymbopogon citratus *(DC) Stapf. which are indigenously used in Kerala, India for curing various infections were tested against a fungus (*Aspergillus niger *) and Gram positive and negative bacteria (*Staphylococcus aureus *and* Escherichia coli*, respectively). Except for* A. marmelos*, all of the aqueous and alcoholic extracts of the plants at 5.0% concentration inhibited the growth of the microorganisms. The diameter (cm) of the inhibition zones of the 5.0% aqueous extracts ranged from 0.8 to 1.6 cm in A.* niger *and 1.0 to 1.4 cm in both* S. aureus *and* E. coli*. . Under the same concentration of alcoholic extracts, the inhibition zones have diameters ranging from 0.9 to 1.8, 1.0 to 1.8, and 1.0 to 2.0 cm for* A. niger*,* S. aureus *and* E. coli *. respectively. Alcoholic extracts of plants were consistently found to be more inhibitory than aqueous extracts of the same concentration. The alcoholic extracts (5.0%) of* L. indica *and* C. citratus *has the highest antifungal activity while* E. odoratum *has the greatest activity against both* S. aureus *and* E. coli*. .*

Key words: *Leucas indica, Cymbopogan citraus, Eupatorium odoratum,* antibacterial activity, antifungal activity, aqueous extract, alcoholic extract

INTRODUCTION

Research in antimicrobial activity of higher plants in India started seriously in the sixties and gained momentum in seventies. A large scale screening of Indian plants for biological activity was conducted by Dhar *et al.*, in 1968. Grainge and Alavarez in 1987 screened about 170 plants for their antimicrobial activity, and found that the leaf extract from *Artobotrys hexapetalus* was inhibitory. Antibacterial and antifungal activities of plant extract were carried out in many laboratories (Ferdous *et al* .,1992, Kodama *et al* .,1993, Amer *et al*., 1994 and Purohit *et al*., 1995). In all the previous studies, attention was given only to a particular family of plant and either to the alcoholic or aqueous extract. The study presents the results of antimicrobial screening of some medicinal plants in Kerala, India using both alcoholic and aqueous extracts from leaves alone or both from leaves and stem.

MATERIALS AND METHODS

Plants were mainly collected from Travancore and Malabar areas of Kerala. These were authenticated by Prof. V.V. Sivarajan (Dept. of Botany. University of Calicut. Kerala). Taxonomic listing of the plants are given in Table 1. Only the leaves and stem of the plants were used. The plant organs were thoroughly washed and dried in shade, ground and 20 g of the powder was successively extracted with double-distilled water and 95% ethyl alcohol in a Soxhlet extractor for 48 h. The aqueous extract was sterilized with Seitz filter. Both aqueous and alcoholic extracts were separately concentrated under reduced pressure. The residue left was weighed and dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 5.0% (w/v). Diluted solutions of 2.5% (2-fold) and 1.25% (4-fold) were made from the original 5.0% solution.

Table 1. List of plants selected for the present study.

No.	Botanical name	Parts extracted
1	*Aegle marmelos* (L.) Corr	Leaves
2	*Leucas indica* (L.) Vatka	Leaves and Stems
3	*Murrya koenigii* (L.) Spreng	Leaves and Stems
4	*Tamarindus indica* (L.)	Leaves
5	*Pachyptera alliacea* (Lam.) A. Gentry	Leaves
6	*Eupatorium odoratum* (L.)	Leaves and Stems
7	*Moringa oleifera* (Lamk.)	Leaves and Stems
8	*Cinnamomum veerum* (Preal.)	Leaves
9	*Cymbapogon citratus* (DC) Stapf.	Leaves

RESULTS

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The bacteria and fungus used in this study were obtained from the Department of Microbiology, Medical College, Calicut, Kerala. These DMSO extracts were screened for their antibacterial activity against *S. aureus* and *E. coli* and antifungal activity against *Aspergillus niger* by disc diffusion test (Maruzzella and Henry, 1958). Nutrient agar and potato dextrose agar were used to culture the bacteria and fungus, respectively . The bacteria were inoculated into nutrient agar broth and incubated at 37°C in a water bath and the suspensions were checked to approximately provide 10^5 cells/ml. From this 200 µl of suspension is transferred in the petri plates containing nutrient agar and the suspension was spread evenly on the medium with a glass spreader to get a uniform lawn of bacteria. For getting a fungal mat, spores of *A. niger* was suspended in 3 to 5 ml of normal saline solution taken in a test tube and the spore suspension was poured over a petri plates containing potato dextrose agar. Excess suspension was drained off. Whatman filter paper disc (No1, 0.5 cm diameter) was placed and test solution of the respective extracts in DMSO (50 µl) were aseptically added with the help of sterile syringes on separate paper discs. Streptomycin and griseofluvin were used as standards for comparison of antibacterial and antifungal activities respectively. These were added in the same manner. Then the plates were incubated at 25°C for fungus and at 37°C for bacteria.. Inhibition was recorded by measuring the diameter of inhibition zone at the end of 24h for bacteria and 72h for fungus. As control, the solvent (DMSO) in which extracts were dissolved was added on separate paper discs. Each experiment was triplicated and the average values are reported in the tables.

RESULTS AND DISCUSSION

All the plants showed antifungal activity at 5.0% of aqueous and alcoholic extracts (Table 2). The aqueous extracts of *M. koenigii*, *C. veerum* and *E. odoratum* showed antifungal activity even in 4- fold dilution . But the aqueous extracts of *M. oleifera*, *A. marmelos* and *P. alliacea* showed antifungal action only in 5.0% solution. *L. indica*, *T. indica* and *C. citratus* did not show any action in their 4- fold dilution (Table 2). Alcoholic extracts of all the plants except *A. marmelos* and *L. indica* showed antifungal activity even in 4- fold dilution (Table 2). Most striking antifungal action was shown by *L. indica*, *C. citratus* and *E. odoratum* . Their antifungal action in 5.0% solution of both aqueous and alcoholic extract was almost same (Table 2). The antifungal action of *C. citratus* , *C. veerum* and *M. koenigii* may be due to their oils and its derivative (Lima *et al* ., 1993, Pandey and Dubey 1994).

Table 2. Diameter (cm) of inhibition zones of the different concentration of alcoholic and aqueous extracts of nine plants on the microorganisms.

Plant name	Con. (%)	Microorganisms					
		A. niger		*S. aureus*		*E. coli*	
		Alcoholic	Aqueous	Alcoholic	Aqueous	Alcoholic	Aqueous
A. marmelos	5.00	0.9	0.8	1.00	1.0	1.00	0.0
	2.50	0.0	0.0	0.00	0.0	0.00	0.0
	1.25	0.0	0.0	0.00	0.0	0.00	0.0
L. indica	5.00	1.8	1.6	1.0	1.4	1.6	1.2
	2.50	1.5	1.0	0.8	0.0	1.4	1.1
	1.25	1.0	0.0	0.0	0.0	1.2	1.0
M. koenigii	5.00	1.4	1.2	1.5	1.0	1.4	1.0
	2.50	1.2	0.8	1.2	0.0	1.1	0.9
	1.25	1.0	0.5	0.8	0.0	0.8	0.0
T. indica	5.00	1.5	1.4	1.2	1.0	1.5	1.0
	2.50	1.0	1.0	1.0	0.0	1.2	0.0
	1.25	0.8	0.0	0.0	0.0	1.0	0.0
P. alliacea	5.00	1.4	1.1	1.0	1.0	1.2	1.1
	2.50	1.0	0.0	0.9	0.0	1.1	0.9
	1.25	0.8	0.0	0.0	0.0	1.0	0.8
C. veerum	5.00	1.2	1.0	1.2	1.0	1.5	1.0
	2.50	1.0	0.8	1.0	0.9	1.2	0.9
	1.25	0.8	0.6	0.8	0.0	1.0	0.0
C. citratus	5.00	1.8	1.6	1.3	1.0	1.6	1.0
	2.50	1.6	0.6	1.0	0.8	1.0	0.9
	1.25	1.4	0.0	0.8	0.0	0.9	0.8
E. odoratum	5.00	1.6	1.5	1.8	1.2	2.0	1.4
	2.50	1.2	0.7	1.5	1.0	1.6	1.1
	1.25	0.8	0.5	1.2	0.8	1.4	1.0
M. oleifera	5.00	1.0	0.8	1.4	1.0	1.4	1.0
	2.50	0.0	0.0	1.2	0.0	1.1	0.0
	1.25	0.0	0.0	1.0	0.0	1.0	0.0

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e plants on the

E. coli	
Alcoholic	Aqueous
1.00	0.0
0.00	0.0
0.00	0.0
1.6	1.2
1.4	1.1
1.2	1.0
1.4	1.0
1.1	0.9
0.8	0.0
1.5	1.0
1.2	0.0
1.0	0.0
1.2	1.1
1.1	0.9
1.0	0.8
1.5	1.0
1.2	0.9
1.0	0.0
1.6	1.0
1.0	0.9
0.9	0.8
2.0	1.4
1.6	1.1
1.4	1.0
1.4	1.0
1.1	0.0
1.0	0.0

A more detailed perusal of results (Table 3) showed that using alcoholic extracts, all the nine plants studied exhibited antifungal activity at 5.0% concentration of the extracts. At 2-fold and 4-fold only two plants namely *A. marmelos* and *M. oleifera* failed to respond. Antibacterial activity against *S. aureus* and *E. coli* was also shown by all the plants at 5.0% concentration. At 2-fold dilution 8 plants retained inhibitory action against both organism. However at 4-fold dilution of extracts only 5 plants were effective against *S. aureus* in comparison to 8 plants with *E. coli*.

Table 3. Number of plants showing inhibition to the microorganisms and the range of diameter (cm) of inhibition zones at different concentrations of alcoholic extracts.

Microorganisms	Concentrations	No. of Plants Total No = 9	Diameter (cm) of inhibition zones
A. niger	5.00	9	0.9 – 1.8
	2.50	7	1.0 – 1.6
	1.25	7	1.0 – 1.6
S. aureus	5.00	9	1.0 – 1.8
	2.50	8	0.8 – 1.5
	1.25	5	0.8 – 1.2
E. coli	5.00	9	1.0 – 2.0
	2.50	8	1.0 – 1.1
	1.25	8	0.8 – 1.4

The responses given by aqueous extracts are given in Table 4. Though all the nine plants inhibit *A. niger* growth at 5.0% level, at 2-fold and 4-fold dilution's only 6 and 3 plants respectively were effective. Against *E. coli*, the responses at 5.0%, 2.5% and 1.25% concentration were exhibited by 8,6 and 4 plants respectively. The most prominent observation was the lack of inhibition of *S. aureus* growth by aqueous extract at lower concentrations. All nine plants showed inhibition of growth at 5.0% and at 2-fold dilution, only 3 showed activity and at 4-fold dilution only one plant was effective.

Table 4. Number of plants showing inhibition to the microorganisms and the range of diameter (cm) of inhibition zones at different concentrations of aqueous extracts.

Microorganisms	Concentrations	No. of Plants Total No = 9	Diameter (cm) of inhibition zones
A. niger	5.00	9	0.8 – 1.6
	2.50	6	0.6 – 1.0
	1.25	3	0.5 – 0.6
S. aureus	5.00	9	1.0 – 1.4
	2.50	3	0.8 – 1.0
	1.25	1	0.8
E. coli	5.00	8	1.0 – 1.4
	2.50	6	0.8 – 1.1
	1.25	4	0.8 – 1.0

The zones of inhibition obtained from 5.0% solution of aqueous and alcoholic extracts of *E. odoratum* is 1.2 cm and 2.0 cm respectively. This corresponds to zone of inhibition obtained for 100 mg of Streptomycin used as standard. Both aqueous and alcoholic extracts of *E. odoratum* showed antibacterial activity against Gram negative bacteria. *Chromolaena odoratum* which belongs to the same family (Copositae) also showed antibacterial action (Irobi, 1992). Four fold dilution of aqueous extract of *P. alliacea* and *C. citratus* showed antibacterial activity against Gram negative bacteria while the 4- fold dilution of *C. veerum*, *M. koenigii* and *M. oleifera* did not have any effect against Gram negative bacteria. The antimicrobial action of *P. alliacea* was also reported by Sharma (1993). The greatest antibacterial activity against

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Diameter (cm) of nhibition zones
0.8 – 1.6
0.6 – 1.0
0.5 – 0.6
1.0 – 1.4
0.8 – 1.0
0.8
1.0 – 1.4
0.9 – 1.1
0.8 – 1.0

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Gram negative bacteria was shown by *E. odoratum* . The antibacterial action of *M. oleifera* and *A. indica* was reported by Mendia *et al* (1991) and Iyer and Williamson (1991).

The study shows that the alcoholic extract has a better antifungal action than aqueous extract. This is in agreement with the report of Islam *et al* (1992). That the antibacterial/antifungal activity of the alcohol extractable fraction was due to more than one active principles was confirmed following TLC separation of components (data not shown). This finding merits further detailed studies as regards the chemical nature of the compounds particularly *L. indica* , *C. citratus* and *E. odoratum* for promising antifungal agents. The use of E. *odoratum* and L. *indica* in Kerala for various skin infections is justified by this work, as it showed commendable activity against both the test organisms.

SUMMARY

L. indica, *C. citratus* and *E. odoratum* has high potentials as an antifungal and antibacterial agents, respectively. The alcohol extractable fraction elicited greater activities than the aqueous extracts from the plants. The presence of one or more active principles is also indicated. Identification of the active principles from these plants must be carried out as they may be new and novel sources of both antifungal and antibacterial compounds.

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Phillipine J of Science(1998), 127(1): 65-72. Sasidharan et al. Antimicrobial activity of nine common plants in Kerala, India.

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BIOLOGICAL ACTIVITY OF SAPONINS FROM TWO *DRACAENA* SPECIES

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ABSTRACT

Many species of the west African "soap tree" *Dracaena* are used in traditional medicine for the treatment of a variety of diseases. In continuation of our search for anti-infective agents from plants implicated in traditional medicine, we evaluated the biological activities of saponins from extracts of *Dracaena mannii* and *Dracaena arborea* by using a battery of test systems such as radiorespirometry, Cytosensor®, bioautography, and agar dilution methods and molluscicidal tests.

Bioassay-directed fractionation of the methanol extracts of seed pulp using a combination of chromatographic techniques, gel filtration, droplet countercurrent chromatography (DCCC), and low-pressure liquid chromatography (Lobar), led to the isolation and characterization of spiroconazole A, a pennogenin triglycoside [3β-*O*-{(α-L-rhamnopyranosyl(1→2),α-L-rhamnopyranosyl(1→3)-β-D-glucopyranosyl}-17α-hydroxyl-spirost-5-ene] (Fig. 1). As the active constituent, spiroconazole A exhibited pronounced antileishmanial, antimalarial, and molluscicidal activities. This paper also reports on the fungistatic, fungicidal and bacteriostatic activity of spiroconazole A against 17 species of fungi and 4 of bacteria.

INTRODUCTION

Available drugs for the treatment of diseases due to various protozoal infections are inadequate due to increasing parasite resistance and serious toxicity associated with some of them. In continuation of our screening program in search of anti-infective agents from plants implicated in traditional medicine, we evaluated the biological activities of saponins from extracts of *Dracaena mannii* and *D. arborea* using a battery of test systems (such as radiorespirometry, Cytosensor®, bioautography, and agar dilution methods, and

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molluscicidal tests). The initial goal of this program was the identification of compounds having antifungal and molluscicidal properties. Previously, we reported one antifungal and several molluscicidal constituents of *D. mannii* (Okunji *et al.*, 1990, 1991).

Because antiprotozoal and antifungal activities are frequently associated with the same or chemically similar compounds, we considered it probable that spiroconazoles, the main saponin constituent of the two species of *Nigerian Dracaena*, would have antiprotozoal activity.

In general, antiprotozoals are not given high priority for commercial development because the per capita health expenditure in many tropical countries is less than the cost of one course of drug therapy. Thus, many "modern" antiparasitic drugs were initially marketed >40 years ago. Clinical intervention in the treatment of leishmaniasis, for example, is presently limited to the use of pentavalent antimonials (SbV), sodium stilbogluconate and *N*-methylglucamine antimonate, and secondarily, amphotericin B, or pentamidine (Croft, 1988; Bryceson, 1987). Treatment with these agents is not consistently effective, particularly for the most virulent leishmanial disease forms (Croft, 1988; Bryceson, 1987; Jha, 1983; Rocha *et al.*, 1980; Mebrahtu *et al.*, 1989). Furthermore, most of the current antiprotozoal drugs are very toxic. It would, therefore, be useful to develop more effective, less toxic, and orally active antileishmanials. The antileishmanial activity of the extracts from the Nigerian plant *Dracaena mannii* has been evaluated by determining their effect on parasite growth and on the catabolism of various substrates using the radiorespirometric microtest, RAM. The *in vitro* RAM, a metabolic test using leishmanial promastigotes (i.e. the monoflagellate extracellular culture forms shown in Fig. 2a), had been developed earlier in our laboratories. The RAM relies on drug inhibition of parasite production of $^{14}CO_2$ from a battery of ^{14}C-substrates to detect drug-mediated parasite damage at low drug concentration within a short time (Jackson *et al.*, 1989, 1990).

Another protozoan disease, malaria, remains the greatest human killer among parasitic infections, despite the world-wide effort to combat the disease and attempts at the eradication of the causative organisms. The emergence of multi-drug-resistant strains of *Plasmodium falciparum*, the most lethal of the malaria parasites, poses a serious health-care problem, not only in the malaria-endemic countries but also among international travellers.

Protozoan infections are also a major cause of mortality and morbidity in immunosuppressed patients, as in acquired immunodeficiency syndrome (AIDS). A single therapeutic agent active against different types of protozoa would be a major innovation in the treatment of these diseases.

Similarly, fungal and yeast infections are becoming increasingly resistant to modern drugs. In immunologically compromised individuals, for example, complications arising from uncontrollable fungal infections are among the leading cause of death. There is, therefore, a need for new and effective alternative treatment. This paper describes and summarizes our investigation of the therapeutic potential of these commonly used medicinal plants using a battery of biologic test systems.

MATERIALS AND METHODS

Plant Materials

Two species of *Dracaena, D. mannii* and *D. arborea*, were collected at Isi-elu, near the Nsukka campus of the University of Nigeria in February, 1985. The collection was chosen from plants listed in an ethnomedicinal survey carried out among the Igbo people (Iwu, 1981/82, 1993). The *Dracaena* spp. plants were taxonomically identified by Mr. A. Ozioko of the Department of Botany, University of Nigeria, Nsukka and the identities confirmed by Dr. J. C. Okafor of the Forestry Herbarium, Enugu. Voucher specimens

have been deposited at the Department of Pharmacognosy Herbarium, University of Nigeria, Nsukka. Prior to extraction, the plant material was dried at 40 °C and the dried vegetable drug ground to coarse powder.

For column chromatography (CC), silica gel 60 size 0.063-0.200 mm (70-230 mesh ASTM, EM Science, was used, and Sephadex LH-20, Sigma, for gel filtration. Low-pressure liquid chromatography (Lobar) was done using a LichroPrep RP-8 column (40-63 mm 2.5 X 25 Merck) equipped with an FMI pump. DCCC equipment consisted of type 300 glass tubes (length 400 mm, I.D. 2 mm) (Tokyo Rikakikai, Nishikawa Bldg, Toyama-Cho, Kanda Chiyoda, Tokyo), solvent system: $CHCl_3$:MeOH:H_2O (7:13:8). The solvent systems for CC were all homogeneous. Thin-layer chromatography (TLC) was used on the Analtech normal phase 10 x 20 cm plates. The TLC plates were developed using solvent systems were: I. $CHCl_3$:MeOH:H_2O (65:40:5), and II. $CHCl_3$:MeOH:H_2O (40:10:1). Sephadex LH-20 gel (25-100 mm size; Sigma) filtration was performed using methanol as eluant.

Extraction and Isolation Protocol

The powdered fruit pulp of the two species of *Dracaena* was Soxhlet-extracted with solvents of increasing order of polarity in two batches, starting with petroleum ether (bp 40-60 °C) (48 h), chloroform (48 h), ethyl acetate (48 h) and methanol (48 h). Each extract was concentrated to dryness *in vacuo* using a rotary evaporator at 40 °C. The isolation and purification of spiroconazole A, B, and C from *D. mannii* have been described elsewhere (Okunji *et al.*, 1991). Briefly, a portion of the methanol extract (20 g) was first partitioned between chloroform-methanol-water mixture (2:2:1) to yield a saponin-enriched lower organic layer which was concentrated to dryness *in vacuo* and lyophilized. Five grams of the active milky-colored fraction were dissolved in a minimum volume of methanol and chromatographed on a Sephadex LH-20 column (2.0 X 50 cm) with methanol as eluant. The flow rate was adjusted to 2.5 ml min-1 and 10-ml fractions were collected. One gram of the crude active saponin fraction was dissolved in 10 ml of a (1:1) mixture of both upper and lower phases of the solvent system chloroform-methanol-water (7:13:8) and then subjected to droplet countercurrent chromatography (DCCC) in the ascending mode. The more polar upper layer was used as the mobile phase. The sample was injected into the apparatus via a 15-ml sample chamber. The flow rate was 10 ml h-1, and the eluates were collected in 5-ml fractions. The monitoring of the fractions was carried out with TLC aluminum sheet silica gel 60-F254 in solvent systems I and II. The saponins were detected with Godin reagent (Godin, 1954). Low-pressure liquid chromatography on a Lichroprep RP-8 (40-63 mm) column was used as the final purification of the saponins. Two molluscicidal spirostanol saponins that we designated as spiroconazole A and B, and a third non-molluscicidal saponin, spiroconazole C, were isolated and characterized on the basis of spectroscopic evidence. Similar phytochemical and biological patterns were observed for *D. arborea*.

Antimalarial Bioassay

The *in vitro* antimalarial assays were performed by using a modification of the semi-automated microdilution technique described earlier (Desjardins *et al.*, 1979, Milhous *et al.*, 1985). Two *Plasmodium falciparum* malaria parasite clones, designated Indochina (W-2) and Sierra Leone (D-6), were utilized in susceptibility testing. The W-2 clone is resistant to chloroquine, pyrimethamine, sulfadoxine, and quinine, and the D-6 clone is resistant to mefloquine. The test compound, spiroconazole A, was dissolved in DMSO and serially diluted using malarial growth medium. Drug-induced reduction in uptake of tritiated hypoxanthine was used as an index of inhibition of parasite growth. In this assay,

Table 1. Antifungal activity of spiroconazole A, compared to current antifungal drugs: miconazole and ketoconazole. Both minimum inhibitory concentration , MIC, and minimum fungicidal concentration (MFC) are given in μg ml^{-1}. Adapted with permission from C.O. Okunji, C.N. Okeke, H.C. Gugnani, and M.M. Iwu, Int. J. Crude Drug Res. 28:193-199, 1990.

	Spiroconazole A		Miconazole		Ketoconazole	
Test Fungi	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
Dermatophytes						
Trichophyton mentagrophytes	12.50	25.00	6.25	25.00	6.25	25.00
Trichophyton tonsurans	12.50	50.00	1.56	6.25	0.78	3.13
Trichophyton soudanense	6.25	12.50	0.20	0.78	0.05	0.39
Trichophyton rubrum	12.50	25.00	3.13	6.25	1.56	6.25
Microsporum audouinii	12.50	25.00	1.56	3.13	0.20	0.39
Microsporum grpseum	50.00	100.00	12.50	100.00	0.30	1.56
Pathogenic Dermatiaceous Fungi						
Phialophora verrucosa (ATCC 50768)	50.00	100.00	0.05	0.20	0.10	0.20
Fonsecaes pedrosoi (ATCC 52593)	25.00	50.00	0.20	0.39	0.05	0.10
Cladosporium carrionii	12.50	12.50	0.10	0.39	0.10	0.30
Cladosporium tennuisimum (ATCC 623337)	100.00	100.00	0.78	3.13	0.39	0.78
Exophiala jeanselmei (ATCC 62791)	25.00	100.00	0.20	0.39	0.10	0.39
Ramichloridium subulatum (ATCC 62339)	25.00	1001.00	0.39	25.00	0.20	0.39
Yeasts						
Candida albicans	25.00	100.00	6.25	6.25	12.50	25.00
Candida tropicalis	100.00	100.00	6.25	6.25	1.56	1.56
Trichosporon cutaneum	6.25	6.25	0.05	0.02	0.02	0.78
Geotrichum candidum	12.50	12.50	1.56	1.56	0.39	0.78
Rhodotorula sp.	25.00	100.00	1.56	1.56	0.78	1.56

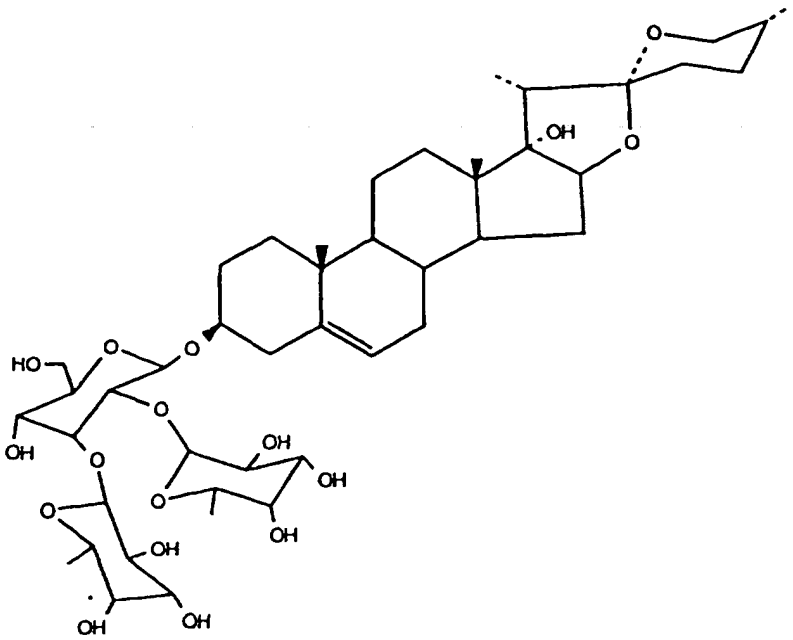

Fig. 1. Chemical structure of spiroconazole A.

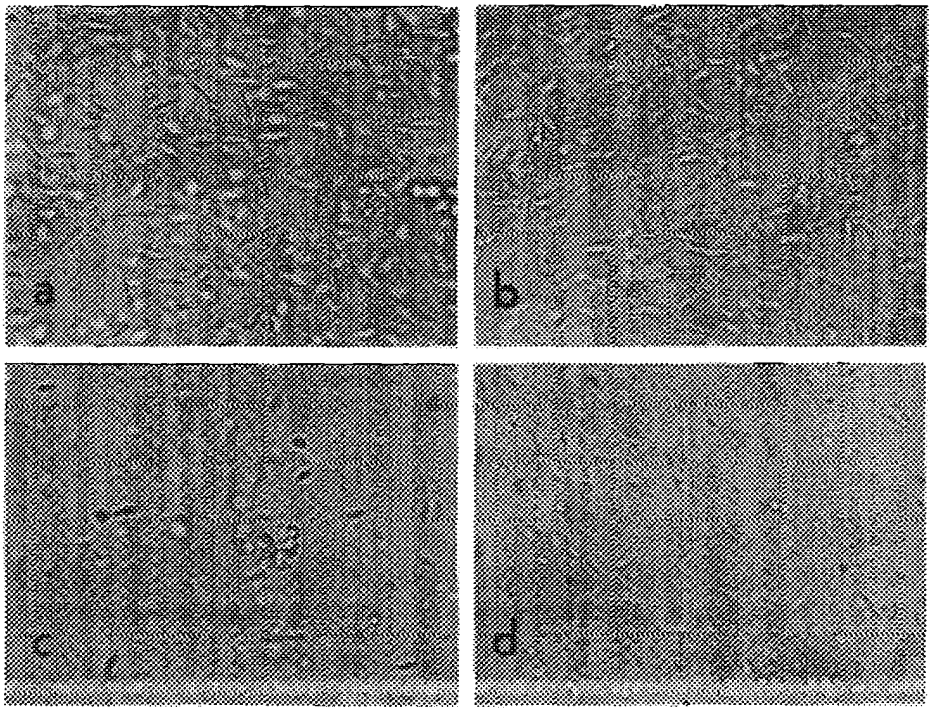

Fig. 2. Photograph showing leishmanial promastigote morphology of control (6a: 0.6% DMSO), and spiroconazole A-treated parasites (6b: 6.3-; 6c: 12.5-, and 6d: 50 μg ml^{-1}) after 17.5 h drug exposure during logarithmic phase growth.

the spiroconazole A treatment resulted in an IC_{50} value of 0.03 μg ml^{-1} for the W-2 clone, and 0.1μg ml^{-1} for the D-6 *Plasmodium falciparum* clone.

Antifungal Tests

TLC Bioassay:

A method similar to that of Homans and Fuchs (1970) was employed in this investigation. This technique involves direct spraying of thin layer chromatograms with conidial suspensions of a test organism. About 100 μg of extract was spotted on silica gel TLC plates and developed with solvent system I. Developed plates were separately sprayed with either a spore suspension of *Cladosporium cucumerinum*, and subsequently with spore suspensions of *Cladosporium carrionii*, *Cladosporium cladosporioides*, *Cladosporium tennuisimum* and *Fonsecaea pedrosoi*, to determine the spectrum of activity. The plates were then incubated in sealed humid chambers at room temperature for four days in the dark. Antifungal activity was manifested by the appearance of a white spot, corresponding to the position of the active compound, surrounded by a grey-black fungal growth all over the plates (Fig. 3). Bioassay-directed fractionation of the active extracts using a combination of chromatographic techniques led to the isolation and characterization of the spiroconazole group of compounds. The most active compound, spiroconazole A, gave a clearly visible inhibition zone at a concentration of 5 μg, which is below the limit of the detecting reagent (Godin's spray reagent).

Agar Diffusion Method:

The dermatiaceous fungi used in this work were environmental isolates (Okeke and Gugnani, 1986) and have been deposited in the American Type Culture Collection (ATCC). Culture accession numbers (designated ATCC#) are indicated in Table 1. The yeasts and dermatophytes were clinical isolates from the University of Nigeria Teaching Hospital, Enugu.

The antifungal activity of spiroconazole A was evaluated by the agar diffusion method using Emmon's Sabouraud dextrose agar (ESDA) as the growth medium. Stock solutions of the test compound and reference standard antifungal drugs, ketoconazole (R41,4001; lot C4,701) and miconazole (ZR-14,889; lot H1001), were prepared at initial concentrations of 10 X 10^3 μg ml^{-1} of dimethyl sulfoxide (DMSO). Serial 2-fold concentrations (0.025-100 μg ml^{-1}) were incorporated into the growth medium and plates were poured. ESDA incorporating only DMSO was used as control. Plates were inoculated with 0.05 ml of the fungal suspensions (approximately 10^5 conidia or hyphal elements/ml 0.9% sterile saline) in triplicate and incubated at 30 °C until macroscopically visible growth appeared in the control (48-96 h post incubation). The minimum inhibitory concentration (MIC) was the lowest concentration of compound that inhibited fungal growth. The minimum fungicidal concentration (MFC) was determined by culturing portions of the fungal inocula of the MIC test plates showing no sign of fungal growth onto fresh plates of ESDA in triplicate. The plates were incubated at 30 °C for 48-96 h. The lowest concentration at which the fungal inoculum yielded no visible growth was taken as the MFC.

In this assay, the most active analog, spiroconazole A, was shown effective against the yeasts and fungi at the drug concentrations listed in Table 1.

In Vitro Antileishmanial Activity

An *in vitro* radiorespirometric microtest (RAM) technique was used to evaluate the spiroconazoles for possible antileishmanial activity. This method, as already noted, relies

on drug inhibition of parasite production of $^{14}CO_2$ from a battery of ^{14}C-substrates by promastigotes to detect drug-mediated parasite damage at low drug concentration within a short time. The test is quantitative, rapid, consistent, and is conducted in serum-free medium in which prior adaptation is not necessary to cultivate the so-called "difficult to grow" species.

Leishmania species/strains:

A clinical isolate of visceral *Leishmania (Leishmania) chagasi*, MHOM/BR/84/BA-13, was used for this study. This isolate was selected because sensitivity to SbV was previously determined using RAM. MHOM/BR/84/BA-13 is sensitive to Pentostam®, sodium antimony gluconate, at 6 μ ml^{-1} Sb (20 μg ml^{-1} drug); and to Glucantime®, *N*-methylglucamine antimonate, at 80 μg ml^{-1} Sb (286 μg ml^{-1}drug).

The ^{14}C-labelled substrates are (numerical codes given in the x-axis of Fig. 4) ^{14}C-substrates: (3) L-aspartic acid (4-^{14}C); (7) glycine (U-^{14}C); (10) L-leucine (1-^{14}C); (13) L-ornithine (1-^{14}C); (25) D-galactose (1-^{14}C); (28) D-mannose (1-^{14}C); (44) succinic acid (1,4-^{14}C); and (46) Na-butyrate (1-^{14}C). All ^{14}C-substrates were selected with specific activities as close to 40 mCi mM^{-1} per carbon atom as obtainable from commercial sources. The quantitative promastigote growth inhibition assay was used as a guide to identify isolates exhibiting antileishmanial activity.

RAM Drug Test Procedure:

The procedure was conducted as previously described (Jackson *et al.*, 1989, 1990). Promastigotes were maintained in log phase growth for 3 successive transfers (48-72 h apart) prior to radiorespirometric (RAM) testing. Test samples (or PBSS, 0.1 M phosphate-buffered balanced salt solution, plus drug solvent, DMSO, for parallel control cultures) was added 24 h after the third promastigote transfer to fresh growth medium. Incubation in the presence of plant samples was continued for 96 additional hours while the parasites remained in mid-log phase growth. The test compound was tested at 50 μg ml^{-1}. Drug sensitivity or resistance was based on ^{14}C-substrate(s) (listed above) for which $^{14}CO_2$ release was decreased for drug-treated parasites compared to parallel tests of phosphate-buffered balanced salt solution and vehicle (PBSS+DMSO) controls. Each experiment consisted of parallel: (a) duplicate tests of drug-treated parasites; plus (b) duplicate tests of drug vehicle control-treated parasites; plus (c) one "nonbiological" sterility control. The nonbiological control consisted of each ^{14}C-substrate (one substrate per microtiter tray well), and PBSS (the same PBSS batch used to wash, to suspend the parasites, and to make drug solution). Since there were no parasites in the nonbiological control, any $^{14}CO_2$ detected was attributed either to biologic contamination (or, less likely, chemical contamination) of the ^{14}C-substrates resulting in breakdown of such substrates. If radioactivity above background (10 dpm) was detected in the nonbiological control, the suspect solution(s) was replaced and the experiment was repeated.

The results (Fig. 5) show that spiroconazole A strongly inhibited the growth of the *Leishmania* strains at the dose of 50 μg ml^{-1}. This test compound also significantly inhibited the leishmanial catabolism of various ^{14}C-substrates, resulting in a maximum suppression of more than 95% when compared with the values observed for the controls (Fig. 4).

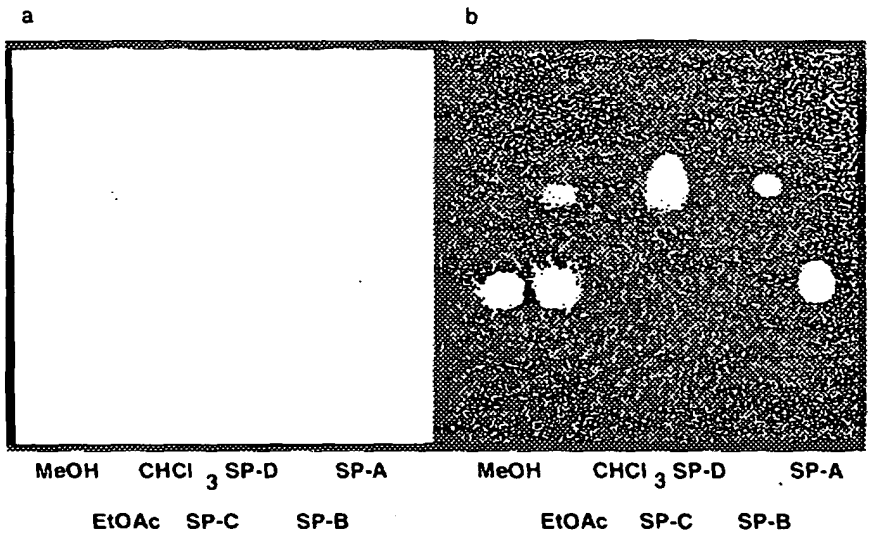

Fig 3. Thin layer chromatography (TLC)-bioassay on a silica gel plate, showing inhibition of the fungus, *Cladosporium cucumerinum*, by *Dracaena mannii* extracts and isolated compounds.

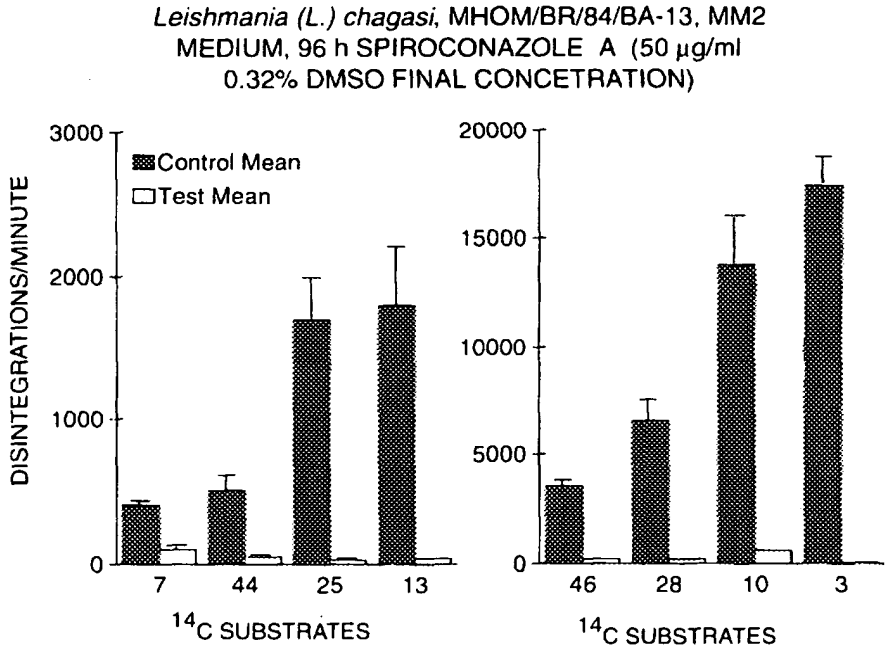

Fig. 4. Radiorespirometric (RAM) data showing markedly reduced respiration of *Leishmania (Leishmania)* chagasi, a visceral disease parasite after spiroconazole A treatment *in vitro*. The vehicle-control-treated parasite respiration is represented by the light grey vertical bars; the spiroconazole A (50 µg ml^{-1} for 96 h)-treated parasites, by the solid black bars. The ^{14}C-substrate numeric codes (x-axis) were given in the corresponding section of the Materials and Methods.

Cytosensor Microphysiometer System

The rate at which cell excrete acids into their environment is closely linked to the rate which they convert food to energy, i.e their metabolic rate. The Cytosensor Microphysiometer System (CMS) measures the rate at which cells acidify their immediate environment. The CMS monitors these metabolic changes as changes in the rate of cellular acidification. In this way, the system provides a real-time, noninvasive means of measuring cellular responses to a wide variety of agents (McConnell *et al.*, 1992).

Spiroconazole A was tested for antileishmanial activity *in vitro* using CMS. Promastigote leishmanial forms were exposed to spiroconazole A in the chemically defined, serum-free medium (Jackson *et al.*, 1989) for 17.5 h during logarithmic growth phase. To prepare cells for CMS, the nonadherent cell protocol was utilized. Briefly, the cells were centrifugally concentrated, counted by hemacytometer, and resuspended in 0.2% low-temperature agarose in balanced salt solution. Leishmanial promastigotes, a 10-µl suspension containing 1-2 X 10^6 cells in agarose, were placed in each of 8 Cytosensor flow-chambers and the low-buffer formulation of RPMI medium (pH 7.4, Molecular Devices Corporation) was pumped over the cells. The repetitive pump cycle time was 2.0 min (88 sec of medium flow followed by 32 sec of pump off). During the 32 sec the peristaltic pump was not operating, the rate of leishmanial acidification of RPMI medium in each of 8 separate cell chambers was measured. Acidification rates during the two-min cycle resulted in less than 0.1 pH unit change and were not detrimental to the leishmanial cells. The CMS leishmanial acidification rates (representative data given in Fig. 6) were relatively constant for each drug treatment concentration (6.3, 12.5, 50 µg ml^{-1}) and vehicle control (0.6% DMSO) duplicate pair, tested in parallel simultaneously, over the 11-h observation period.

In Vivo Antileishmanial Activity

The *in vivo* antileishmanial activity was determined by administering various doses of the spiroconazole A to golden hamsters and determining the effect on laboratory-induced visceral and cutaneous leishmaniasis of the animals. For this assay, the compounds were tested against *Leishmania (Leishmania) donovani*, MHOM/SD/43/Khartoum, a causative organism of kala azar or visceral leishmaniasis, and *Leishmania (Viannia) panamensis*, MHOM/PA/83/WR539, an etiological agent of simple cutaneous leishmaniasis. Spiroconazole A was tested in each *in vivo* leishmanial model by the oral, intramuscular, and subcutaneous routes of administration.

The results of the activity of the spiroconazole A administered through the intramuscular route to hamsters infected with cutaneous *L. panamensis* represent an example of dose-dependent *in vivo* activity of the compound. At a dose of 104 mg kg^{-1} total dose (equivalent to 26 mg kg^{-1} per day) of the spiroconazole A, administered by intramuscular route twice a day for 4 days, the test substance produced a 73% inhibition of lesion caused by *L. panamensis* in hamsters. A dose of 52 mg kg^{-1} (13 mg kg^{-1} per day) by the same regimen gave a 51% reduction of the lesion area, and at a dose of 13 mg kg^{-1} (3.25 mg kg^{-1} per day) 7% reduction of the lesion area was observed.

Antibacterial Activity:

Antibacterial activity of spiroconazole A was evaluated by the agar well assay method using trypticase soy agar (Difco) as the growth medium. Plates of this medium were inoculated with 0.1 ml of a 6th culture of the test isolate in trypticase soy broth, a sterile glass spreader being used to ensure uniform growth of the inoculum. Wells (10 mm diameter) were made in the seeded agar plates and 0.1 ml of 1% solution of spiroconazole

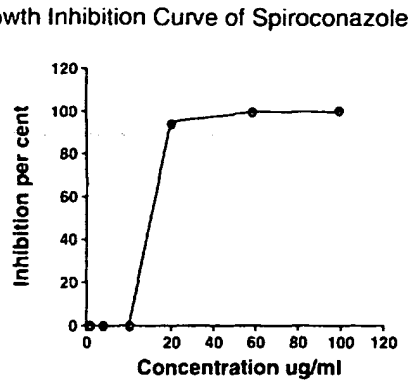

Fig. 5. Growth inhibition (y-axis) for *Leishmania (Leishmania)* chagasi with increasing spiroconazole A concentration (x-axis).

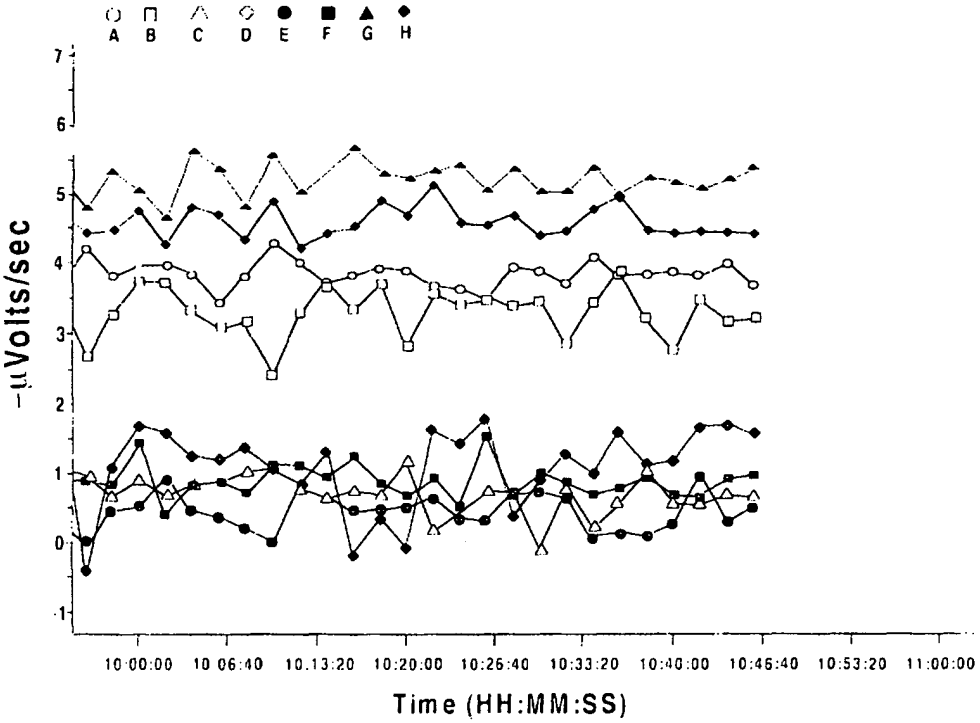

Fig. 6. Cytosensor microphysiometer (CMS) antileishmanial promastigote results after 17.5 h spiroconazole A treatment. The duplicate control parasite (i.e. parasites treated with drug solvent, 0.6% DMSO) tests, represented as uppermost lines, "G" and "H", have a consistently higher metabolic rate during the 11 h of observation. Parasites preincubated in parallel with controls for 17.5 h with 6.3- (lines "A" and "B"). 12.5- (lines "C" and "D"), and 50 µg ml^{-1} spiroconazole A (lines "E" and "F"), manifest lower metabolic rates, with the two highest drug concentrations resulting in metabolic rates very close to zero.

A in DMSO was introduced into the wells in triplicate. Streptomycin at a concentration of 100 μg ml^{-1} was used as reference standard and 0.1 ml DMSO as a control. The plates were incubated at 37 °C and the diameter of zones of inhibition was measured across each well after 24 h. The MIC for bacteria was determined in trypticase soy broth to which were added serial 2-fold concentrations (0.025-200 μg ml^{-1}) of spiroconazole A. The tubes were inoculated in triplicate with 0.01-ml quantities of 6th broth cultures of the test isolates. The tubes were incubated at 37 °C for 24 h and examined spectrophotometrically at 530 nm. The lowest drug concentration that showed no turbidity was taken as the MIC. Streptomycin was used as the standard reference drug.

Molluscicidal Potency Test

Two local snail vectors; *Bulinus globosus* and *Biomphalaria pfeifferi*, were collected from a pond near Nkalagu Cement Factory in the Isielu Local Government Area of Enugu State, Nigeria and reared in our laboratory. Living snails were identified to species by the staff of the Department of Zoology, University of Nigeria. The residue from methanol extracts of *Dracaena* fruit pulp and spiroconazole A were separately dissolved in distilled water. This was made into a stock solution of 100 ppm before serial dilution to obtain desired concentrations. Molluscicidal tests were carried out according to Duncan and Sturrock (1987) using laboratory-reared snails. Tests were carried out in two replicates per test compound concentration. Ten snails (6-10 mm in height) were exposed for 24 h allowing 24 h for the recovery period after which mortality rate was determined. Tests to evaluate the effects of physicochemical factors (UV and pH) on the molluscicidal activity of spiroconazole A were carried out as described by Adewunmi and Marquis (1980).

RESULTS AND DISCUSSION

In a first activity-directed investigation, the methanol extracts of the fruit pulp of *D. mannii* and *D. arborea* exhibited strong antifungal and molluscicidal activities. Bioassay directed fractionation of this active fraction led to the isolation of a spiroconazole group of compounds. The antifungal activity of extracts of these plants was originally detected by direct spraying of TLC plates with a spore suspension of the test fungus *Cladosporium cucumerinum*. A clearly visible inhibition zone, even at the lowest concentration of 5 μg, was observed after using spiroconazole A (illustrated in Fig. 3). This concentration is below the detectable limit of the freqently used spray reagent (Gódin, 1954) for saponins.

Spiroconazole A was tested for fungistatic, fungicidal and bacteriostatic activity against 17 species of fungi (results summarized in Table 1) and bacteria. These fungi, with the exception of *Cladosporium tennuisimum* and *Ramichloridium subulatum*, are well known either as strict or opportunistic pathogens of humans. The dermatophytes, causal agents of infections of hair, nail and skin, were inhibited at concentrations of 50 μg ml^{-1} or less, with Trichophyton soudanense manifesting greatest sensitivity to the drug (MIC; 6.25 μg ml^{-1}). The MICs for the species of pathogenic dermatiaceous fungi, causal agents of cutaneous and subcutaneous mycoses, were within the range 12.5-100 μg ml^{-1}. All the test yeasts species were inhibited at 100 μg ml^{-1} concentration or less, the most sensitive being Trichosporon cutaneum (MIC, 6.25 μg ml^{-1}). The minimum fungal concentrations were mostly 1-4 times the MIC values. The control antimycotics, ketoconazole and miconazole, commonly used in chemotherapy, showed lower MICs and MFCs relative to the test compound (Table 1). The result of the antibacterial test showed that spiroconazole A was selectively bacteriostatic against the gram-positive bacteria species at 10 X 10^3 μg ml-1 in

the agar assay method. In this study no antibacterial activity was observed at 200 μg ml^{-1} saponin in the MIC assay.

Spiroconazole A possesses strong molluscicidal activity against all the snail vectors. At 5 ppm concentration it exhibited 100% mortality within three h against four species of snails *Bulinus globosus, Bulinus forskalii, Biomphalaria pfeifferii,* and *Lymnaea natalensis,* while *Biomphalaria glabrata* were less susceptible to the 5 ppm lethal dose. However, spiroconazole A at 6 ppm yielded a 100% kill within 24 h against *Biomphalaria glabrata.* It is worthy of note that *Lymnaea natalensis,* which transmits the economically important major animal disease, fascioliasis, is killed within 3 h at 5 ppm lethal dose by spiroconazole A.

The results of the RAM test for leishmanial parasites are given in Fig. 4. After a 96-h incubation with spiroconazole A, no live parasites were observed in culture and RAM respiratory rates for all ^{14}C-substrates reflect this lack of parasite viability. The metabolic rate for every ^{14}C-substrate by the spiroconazole-treated parasites is near zero (solid black bars). The drug-treated results are in sharp contrast to the vehicle control (0.6% DMSO) treated promastigote ^{14}C-substrate catabolism, which show high respiratory rates during the 30-min test period (solid grey bars).

The results using the Cytosensor (Fig. 6) agree well with visual observation of the parasites by light microscopy given in Fig. 2, and the growth inhibition curve, Fig. 5. The vehicle control parasites, Fig. 2a, manifest the typical spindle-shaped monoflagellate form of leishmanial promastigotes. Cell density of the control parasites in culture was 5 X 10^7 ml^{-1}. Motility of the parasites was virtually 100%. Figure 2b shows parasites treated for 17.5 h at 6.3 μg ml^{-1} spiroconazole A. It is evident that at 6.3 μg ml^{-1} drug there are fewer parasites, about half that of the control culture, or 2.5 X 10^7 ml^{-1}, representing marked growth inhibition by spiroconazole A. At 12.5 μg ml^{-1} drug, Fig. 2c, the few remaining parasites are swollen, granulated, and the cytoplasm appears transparent, possibly indicating loss of membrane integrity with cytoplasmic leakage. Little to no motility was seen in parasites treated with 12.5 μg ml^{-1} spiroconazole A, and parasite number in culture was only 5 X 10^5 ml^{-1}. At 50 μg ml^{-1} drug, Fig. 2d, no intact parasites are visible, only hollow parasite membranes, with no cytoplasm. Likewise, an IC_{50} of approximately 10 μg ml^{-1} was observed for the growth inhibition data, Fig. 5. Maximum achievable serum level for SbV drugs, current "drugs-of-choice" for antileishmanial therapy, has been determined to be 20 μg ml^{-1} 1-2 h post-administration (references reviewed in Jackson, *et al.*, 1989, 1990).

Comparative analyses of the polar extracts from *Dracaena* species demonstrated that the spiroconazole analogues are the major biologically active components. These biological effects can perhaps explain the traditional use of the these plant species in treating different skin diseases.

The yield of biologically active saponins in *Dracaena* species is very high, estimated at up to 30% of the fruit pulp. The highest potency levels are localized in the fruit pulp and the molluscicidal material can be produced on a pilot scale. *Dracaenas* are propagated by seed or vegetatively by stem cutting and are drought resistant. Furthermore, the plant is abundant in west Africa (Keay *et al.*, 1964, Hutchinson and Dalziel, 1958) and is well known to the local population as a medicinal plant. The ease of cultivation of this plant will be a positive advantage over better known saponin-producing plants such as endod. The demand for steroid-based drugs such as cortisone and other corticosteroids, sex hormones, cardiotonic glycosides, oral contraceptives has steadily increased. Steroids of plant origin constitute a major part of the raw material for the preparation of such drugs. There is no doubt that the high yield of steroidal saponin from *Dracaena* spp. may serve as starting material for the manufacture of steroids of therapeutic interest.

In conclusion, we have shown broad spectrum activity for spiroconazole A, having antibacterial, antifungal, antimalarial, antileishmanial, and molluscicidal properties. The drug concentration at which this compound acts compares very favorably with drug activity levels for current modern antibacterial, antifungal, antiparasitic, and molluscicidal drugs.

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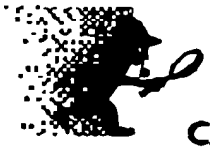

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ANTIMICROBIAL ACTIVITY OF *EUPATORIUM ODORATUM* EXTRACTS

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SUMMARY

Eupatorium odoratum *extracts showed in vitro antimicrobial activity against* Bacillus subtilis, Escherichia coli, Staphylococcus aureus *and* Aspergillus niger. *The chloroform extract displayed maximum inhibitory activity on all microorganisms when compared with the other extracts. Isosakuranetin and kaempferide were isolated from the most active fraction. Lupeol, β-amyrin, betulctol, sakuranetin, 3,5,7,3'-tetra-O-methyl quercetagetin, quercetin and two flavonoid glycosides based on sakuranetin and isosakuranetin moieties were isolated from the other extracts.*

Eupatorium odoratum L. (Compositae) is the only species of *Eupatorium* presently growing in Nigeria. It is distributed widely along the coastal basins of southern Nigeria and is extensively used in Nigeria ethnomedicine as a topical application to arrest bleeding and promote healing. The plant is also an ingredient in the preparation of antimalarial mixtures and cough suppressants.

The main constituents of *E. odoratum* (from India) are 2'-hydroxy-4,4',5',6'-tetramethoxy chalcone (odoratin), the rarely occurring flavone salvigenin and the triterpene alcohols lupeol and β-amyrin[1].

Available literature indicates that no previous antimicrobial study has been done on *E. odoratum* and there is no report on the chemical constituents of the local variety of this plant which appears a new comer in the West African vegetation. We have investigated the major constituents of *E. odoratum* leaf extracts and we report here on the antimicrobial activity of these isolates.

EXPERIMENTAL

Plant material - The leaves of *E. odoratum* were collected in September, 1980 from plants growing in Nsukka Campus of the University of Nigeria. A voucher specimen has been deposited at the Pharmacy Herbarium of the University of Nigeria, Nsukka.

Test organisms - *Escherichia coli* NCTC 9001, *Bacillus subtilis* NCTC 3601, *Staphylococcus aureus* NCTC 3761 and *Aspergillus niger* (laboratory strain).

Microbial methods - The first three organisms listed above were maintained on nutrient agar slopes. Prior to the experiment they were activated by a series of subcultures at a temperature of 37° C with the time of incubation of the spore formers extended to allow for sporulation. The last broth culture in each case was centrifuged, washed and resuspended in sterile saline. Five drops of appropriate dilutions of each leaf extract and each solvent employed for extraction were used for the antimicrobial tests using the cylinder method. Each assay was performed in triplicate.

1 *Phytochemical methods.* Preliminary chemical tests for organic compounds groups were as outlined by Harborne[2].

Extraction and separation. 4 kg of powdered plant material were extracted successively with petrol, $CHCl_3$, Me_2CO, absolute EtOH and H_2O. For the microbial tests the extracts were evaporated to dryness and reconstituted to the appropriate concentrations from which further dilutions were made.

The residue from the petrol extract (80 g) was chromatographed over Si gel column and eluted with petrol - EtOAc mixtures to yield lupeol, β-amyrin and isosakuranetin (5,7-dihydroxy-4'-methoxyflavone). The $CHCl_3$ extract (66 g) was similarly treated and gave kaempferide (3,5,7 trihydroxy-4'-methoxyflavone) and betuletol. The residue from the Me_2CO and EtOH extracts were bulked (250 g) and separated by preparative TLC to give sakuranetin (5,4'-dihydroxy-7-methoxyflavone), 3,5,7,3'-tetra-O-methyl-quercetagetin and quercetin. The aqueous extracts (45 g) was chromatographed on polyamide plates to yield sakuranetin-7-O-arabinoside and isosakuranetin-rhamnoglucoside. The compounds were identified from their spectral characteristics and direct chromatographic comparison (where possible) with reference samples.

Effect of pH on the activity of the extracts. The aqueous extract was acidic (pH5) on concentration and the activity of the extract was also tested after adjusting the pH to 2, 7 and 10 with the appropriate buffer solutions. Blank buffer solutions without any extract were tested at various pH range.

RESULTS AND DISCUSSION

The *E. odoratum* extract exhibits antimicrobial effect as shown in Tab. 1. The chloroform extract exerts maximum inhibitory responses on all the microorganisms tested, whereas the absolute alcohol extract has the lowest, and the activities of the other extracts depend on the microorganisms.

Extract	*S.aureus*	*E.coli*	*B.subtilis*	*A.niger*
Petrol	13	12.5	15	–
Chloroform	15	19	19	11
Acetone	14	14.5	15.5	15
Absolute alcohol	10	10	10	–
Water extract (pH 2)	17	16	15	–
Water extract (pH 8)	13	12	10.5	–
Water extract (pH 10)	9	–	–	–
Control (no drug) pH 1	+	+	+	–
Control (no drug) pH 4	+	+	+	–
Control (no drug) pH 5	–	–	–	–
Control (no drug) pH 6.8	–	–	–	–
Control (no drug) pH 7.5	–	–	–	–
Control (no drug) pH 10	+	–	+	+

(–) = no antimicrobial action; (+) = antimicrobial activity

Tab. 1 - Antimicrobial activity (diameters of inhibition in mm) of *E. odoratum* leaf extracts (1% concentration).

The aqueous extract (pH 5) showed activity on all the organisms except *A. niger*. The extract was tested at pH values of 2, 3, 7, and 10. At pH 7 it has no antimicrobial effect while at pH2 it exerts a maximum effect in comparison to the others. Since pH changes affects the growth of microorganisms[3], control experiments with buffer solutions alone were performed in order to assess the effect of pH alone in the growth of the test organisms. Solutions buffered at pH 5-8 have no effect on microbial growth while at pH 4 or less only the growth of *A. niger* was observed. At high alkalinity (pH 10), the growth of all the organisms was inhibited except *E. coli*. The antimicrobial activity of the aqueous extract of *E. odoratum* does not appear to be due to the acidity of the extract since buffered solutions at the same pH 3 gave no inhibition.

The antimicrobial activity of *E. odoratum* leaf extracts may be due mainly to the presence of flavonoids, and sesquiterpene lactones which are the chief constituents of the leaf, as well as to tannins which occur in small quantities. Flavonoids exert antimicrobial activity in the healing of wounds and in the treatment of skin diseases, quercetin isolated from this plant has been shown to have specific activity on human Herpes-virus[4]. Tannins act as astringent and antibacterial agents. The antiseptic property of tannins is utilized to a certain degree in the treatment of diarrhoea. The presence of tannins in the intestine lowers the multiplication of microorganisms and partly eliminates toxins. A tanned material is quite resistant to bacterial attack[5].

Due to insufficiency of isolated compounds, it was not possible to establish the compounds responsible for the antimicrobial activity. Work is however continuing in our laboratory to isolate the compounds in greater quantity and subject them to antimicrobial tests.

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Mechanism of Hemostatic Activity of *Eupatorium odoratum*

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ABSTRACT

The pressed extract of the leaves of *Eupatorium odoratum* is popularly employed traditionally to arrest bleeding from cuts and for wound dressing. Preliminary investigations showed that the leaf extract of *E. odoratum* significantly reduced bleeding time in guinea pigs and rabbits. The effect was traced largely to its vasoconstrictor activity similar to that of adrenaline. *In vitro* studies showed that the extract concentration-dependently contracted both the rat and guinea pig vasa deferentia and rabbit arterial strips while having no effect on isolated guinea pig ileum and rat stomach strip preparations. The extract-induced contractions were blocked by low concentrations of prazosin but not by atropine, propranolol, mepyramine or pimoxide. These results suggest that the hemostatic action of *E. odoratum* may be partly due to α-receptor mediated vasoconstriction.

Eupatorium odoratum L. (Compositae) is a perennial flowering shrub which grows along the forest zone of West Africa. The plant is widely used in traditional medicine in Nigeria for the treatment of various ailments. In folk medicine the aqueous leaf extract of the plant is used as an antiseptic for wound dressing, while the decoction of the leaf is also commonly used as cough remedy. Singha (1965) reported that the leaf or stem decoction of the plant can be used in pulmonary hemorrhage. In the eastern states of Nigeria, the leaves of *E. odoratum* are commonly used as a hemostatic agent to arrest bleeding from fresh cuts and to stop nosebleeds. Preliminary investigations (Akah 1989) show that the leaf extract of *E. odoratum* significantly reduced bleeding time in guinea pigs and rabbits – an effect traced largely to its vasoconstriction activity. The present study was designed to further explore the mechanism of the hemostatic activity of the aqueous leaf extract of *Eupatorium odoratum*.

MATERIALS AND METHODS

The plant material was collected in October 1987 in Nsukka (Nigeria), in old farm lands within the University campus. Botanical identity was kindly confirmed by Mr. P. Ozioko of Botany Department (Herbarium section). A voucher specimen is deposited in the Pharmacy Herbarium of the University of Nigeria, Nsukka, Nigeria.

Extraction

Fresh leaves of the plant were completely washed with water, spread in shade to dry and then ground to a powder. The powder was soaked in distilled water in a ratio of 100 g to 1000 ml, and the mixture was left for 24 hours. Next, the materials were filtered and the filtrate lyophilized to obtain the extract as solid material.

Pharmacological studies on isolated tissues

Male rats (200-250 g) and male guinea pigs (300-400 g) were killed by a blow on the head and exsanguinated. Pairs of vasa diferentia were removed free from connective tissues and hypogastric nerves. Segments of the ileum and stomach strips were prepared from the guinea pigs and rats respectively. The preparations were set up longitudinally in a 20 ml organ bath containing Tyrode's solution and gassed with air.

For arterial strip studies, adult rabbits of Dutch White breed obtained from the rabbitry of the Deparument of Animal Science of the University were used. Those weighing between 1.5-2 kg were sacrificed by a blow on the head and exsanguinated. A segment of the thoracic aorta between the arch and the diaphragm was removed, cleaned and helically cut strips were prepared as described by Furchgott (1960). Segments of the strips (about 2 mm x 20 mm) were set up in 20 ml organ bath containing normal Ringer solution and gassed with 5% CO_2 in Oxygen. The solutions were maintained at 36°C. The tissues were equilibrated for 60 min during which the bathing solution was changed every 10 min. At the end of the initial equilibration period, responses were established noncumulatively for the extract. Antagonists (prazosin, pimoxide, mepyramine, propranolol and atropine) were allowed 30 min to equilibrate with the muscles before re-establishing response to the extract. Responses were recorded on a Ugo Basile microdynamometer recorder using a 1 g isotonic transducer.

Results were expressed as means and standard errors. The significance of difference between means was determined by Student's t-test and results were regarded as significant when $p < 0.05$.

RESULTS

The extract (1-32 mg) produced concentration-dependent contraction of the isolated rat and guinea pig vasa deferentia given ED_{50} values of 2.75 mg and 5.75 mg, respectively (Fig. 1). Also the extract potently contracted the rabbit arterial strip preparation (Fig. 2). The extract had no remarkable effect on the guinea pig ileum and the rat stomach strips. The extract-induced contractions were not affected by pimoxide, propranolol, mepyramine or atropine in concentrations well above that which will affect their respective specific agonists. The contractions were, however, abolished by prazosin (1.1×10^{-8} - 1.1×10^{-7}M Figs. 2 and 3). Responses to the extract were fully restored in about 15 min. after washing off prazosin.

DISCUSSION

The results of the present study show that the aqueous leaf extract of *Eupatorium odoratum* contains substance(s) that have a potent excitatory effect on the rat and guinea pig vasa deferentia and rabbit arterial strip. The innervation of the vas deferens is noradrenergic (Bentley and Sabine 1963, Birminghan and Wilson 1963, Evans *et al.* 1973, Wadsworth 1973, 1974). Apart from noradrenaline, other sympathomimetic amines such as tyramine have been shown to contract the vas deferens by stimulating alpha adrenoceptors (Barnett *et al.* 1968, Pennefather *et al.* 1974). The presence of excitatory dopaminoceptors has been demonstrated in the rat and guinea pig vas deferens (Tayo 1979). The ability of the alpha adrenoceptor antagonist (prazosin) to abolish the extract-induced contraction of the vas deferens and arterial strips at low concentrations suggests that

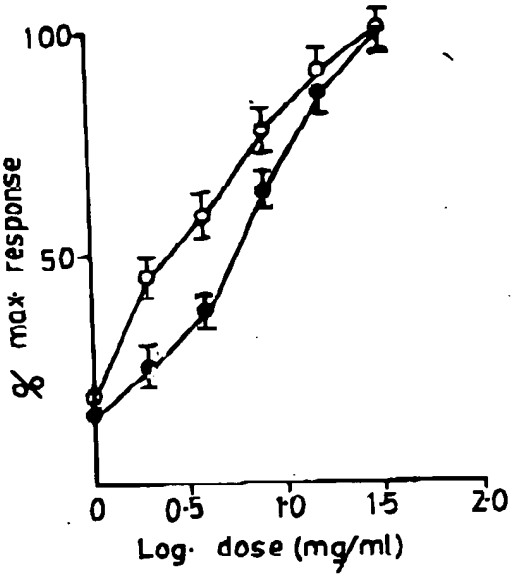

Fig. 1. The extract-induced responses of the rat (–O–) and guinea pig (–●–) isolated vas deferens. Each point represents the mean ± SE of 5 observations.

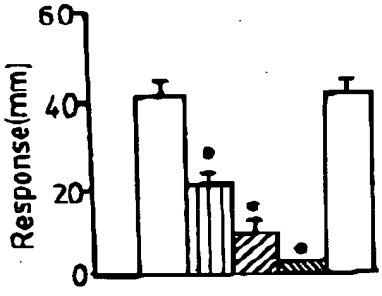

Fig. 2. Effect of prazosin on the extract-induced contraction of the arterial strip.

control

prazosin (1.1×10^{-9} M),

prazosin (1.1×10^{-7}M),

prazosin (1.1×10^{-6}M);

Each bar is the mean ± SE of 5 observations $^{*}P < 0.05$.

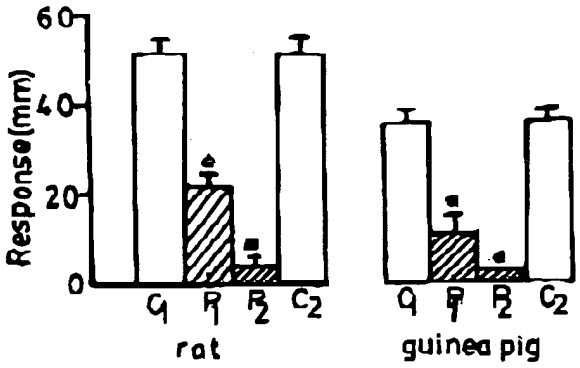

Fig. 3. Inhibition of the extract (32 mg)-induced contractions of the vas deferens by prazosin.
C_1 control response in absence of prazosin
P_1 denotes response in the presence of prazosin (1.1×10^{-8}M)
P_2 denotes response in the presence of prazosin (1.1×10^{-7}M)
C_2 denotes response. 15 min after washing of prazosin ($n = 5$ * $P < 0.05$).

the effect of the extract may be mediated directly or indirectly via alpha adrenoceptors. It may as well be inferred that the potent hemostatic activity of *E. odoratum* may be accounted for by the alpha adrenoceptor mediated vasoconstriction – a property well established for adrenaline. Though several constituents were present in the extract, it is not known with certainty which of them is actually responsible for the observed effects. The identification and isolation of the active substance(s) are subjects of further investigation.

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